UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

Date: September 20, 2019

SUBJECT: **Ethoxyquin:** New and updated DERs for guideline and non-guideline toxicity studies

PC Code: 055501
Decision No.: 549718
Petition No.: N/A
Risk Assessment Type: N/A
TXR No.: 0057942
MRID No.: See Table 1

DP Barcode: D453268
Registration No.: N/A
Regulatory Action: N/A
Case No.: N/A
CAS No.: 91-53-2
40 CFR: §180.178

FROM: Odbert Triplett
Risk Assessment Branch VII,
Health Effects Division (7509P)

THROUGH: Michael S. Metzger, Chief
Risk Assessment Branch V/VII
Health Effects Division (7509P)

TO: Brian Van Deusen, Risk Assessor
Risk Assessment Branch IV,
Health Effects Division (7509P)

I. ACTION REQUESTED:

During registration review, three mutagenicity studies were identified as having previously not received formal reviews in the toxicity database. The ethoxyquin risk assessor requested DERs be composed for these mutagenicity studies to support the ethoxyquin registration review.

II. BACKGROUND:

New DERs were composed for guideline mutagenicity studies: *In Vivo* Mammalian Cytogenetics (MRID 46338501), and *In vitro* Bacterial Gene Mutation (MRID 46330701),and an *In vitro* Mammalian Cytogenetics (MRID 46338901).

III. RESULTS/DISCUSSION:

Table 1. Citation and MRIDs for guideline mutagenicity studies.

Citation	MRID
G.L. Erexson. 2004. *In Vivo* Mouse Micronucleus Assay. Covance Laboratories Inc., Vienna, VA. Study # 7529-110. July 1, 2004. Unpublished.	46338501
Author Michael S. Mecchi, MS (26 July 2004). *Salmonella typhimurium; E. coli* Reverse Mutation gene mutation assay. Covance Laboratories Inc. 7529-102, 26 July 2004. Unpublished	46330701
H. Murli. 2004. Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells. Covance Laboratories Inc. Vienna, VA. Study # 7529-109. August 6, 2004. Unpublished .	46338901

IV. CONCLUSIONS:

For the listed studies the following results were found and DERs were compiled and added to toxicity database.

***In Vivo* Mammalian Cytogenetics (MRID 46338501):** Ethoxyquin appears to be negative for micronuclei induction in mouse bone marrow cells up to dose levels that are overtly toxic for this study. This study was classified as **Acceptable/Guideline** and satisfies the guideline requirement (OCSPP 870.5395; OECD 474) for *in vivo* cytogenetic mutagenicity data.

***In vitro* Bacterial Gene Mutation (MRID 46330701):**There was no evidence of induced mutant colonies over background due to exposure to ethoxyquin up to cytotoxic concentrations observed during this study. This study is classified as **Acceptable/Guideline** and satisfies the guideline requirement (OCSPP 870.5100; OECD 471) for *in vitro* mutagenicity (bacterial reverse gene mutation) data.

***In vitro* Mammalian Cytogenetics (MRID 46338901):** There was evidence of chromosome aberration induced over background. The test article, ethoxyquin, is considered positive for inducing structural chromosomal aberrations in CHO cells in the presence and absence of metabolic activation. This study is classified as **Acceptable/Guideline** and satisfies the guideline requirement (OCSPP 870.5375; OECD 473) for *in vitro* cytogenetic mutagenicity data.

EPA Reviewer: Odbert Triplett **Signature:**
RABVII, Health Effects Division (7509P) **Date:** 09/18/2019
EPA Secondary Reviewer: Austin Wray **Signature:**
RAB IV, Health Effects Division (7509P) **Date:** 09/18/2019

Template version 09/11

TXR#: 0057942

DATA EVALUATION RECORD

STUDY TYPE: *In Vivo* Mammalian Cytogenetics - Erythrocyte Micronucleus assay in Crl:CD-I®(ICR)BR mouse bone marrow; OCSPP 870.5395 [§84-2]; OECD 474.

PC CODE: 055501 **DP BARCODE:** D453268

TEST MATERIAL (PURITY): Ethoxyquin (98.9%)

SYNONYMS: 6-Ethoxy- l,2-dihydro-2,2,4-tri methylquinoline,
1-2-Dihydro-6-ethoxy-2,2,4-trimethl quinoline

CITATION: G.L. Erexson. 2004. *In Vivo* Mouse Micronucleus Assay. Covance Laboratories Inc., Vienna, VA. Study # 7529-110. July 1, 2004. MRID 46338501. Unpublished.

SPONSOR: Pear Bureau Northwest, 4382 S.E. International Way
Suite A, Milwaukie, Oregon 97222-4635 United States of America

EXECUTIVE SUMMARY: In a Crl:CD-I®(ICR)BR mouse bone marrow micronucleus assay (MRID 46338501), ethoxyquin (98.9% a.i., MRI Ref # 8298: 19-30) was administered via gavage to 6-18 male mice/dose at dose levels of 0, 375, 750, and 1500 mg/kg bw. The vehicle used was corn oil. Bone marrow cells were harvested at 24- and 48-hours post-treatment.

A dose range-finding study was conducted prior to the micronucleus assay to inform dose selection in the definitive study. The same ethoxyquin product was administered via gavage to 3 mice/sex/dose at dose levels of 500, 1000, 1500 (males only), and 2000 mg/kg bw. Mortality was observed in 2 males and 2 females at 2000 mg/kg. The remaining 2000 mg/kg animals were humanely sacrificed due to excessive toxicity in the dose group (hypoactivity, squinted eyes, spasms, irregular respiration, recumbency, ataxia, hunched/flattened posture, crust-orbitals, and/or rough haircoat). Males exposed to 1500 mg/kg exhibited some of the same clinical signs of toxicity (squinted eyes, hypoactivity, flattened posture, irregular respiration, crust-orbitals- and rough coat) and a few animals from the 500 and 1000 mg/kg treatment groups (both sexes) exhibited minor clinical signs (slight hypoactivity and squinted eyes) 1 hour after dosing that resolved within a day. No mortalities were observed at doses ≤1500 mg/kg.

In the definitive micronucleus assay, three mortalities were observed in the 1500 mg/kg treatment group and signs of clinical toxicity (hypoactivity, squinted eyes, irregular respiration, ataxia. and/or a temporary trance (standing on hind limbs with head in upright position and little movement)) in the treated animals at 750 and 1500 mg/kg. Ethoxyquin did not induce

statistically significant increases in micronucleated PCEs at any test article dose examined (375, 750, and 1500 mg/kg). The positive control induced the appropriate response. Ethoxyquin was not cytotoxic to the bone marrow at any dose of the test article.

Ethoxyquin is negative for micronuclei induction in mouse bone marrow cells up to dose levels that are overtly toxic.

This study is classified as **Acceptable/Guideline** and satisfies the guideline requirement (OCSPP 870.5395; OECD 474) for *in vivo* cytogenetic mutagenicity data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS:

A. MATERIALS:

1. Test material:

Ethoxyquin

Description: Transparent, viscous, orange liquid

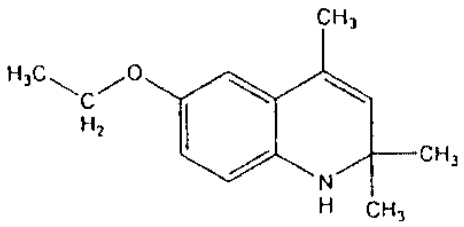
Lot/Batch/MRI #: 8298:19-30

Purity: 98.9% a.i.

CAS # of TGAI: 91-53-2

Solvent Used: Corn oil

Structure:



2. Control materials:

Negative control (if not vehicle) :	Only tested vehicle control	
Vehicle Control: Corn oil	Final volume: 10 ml/kg	Route: Oral Gavage
Positive control : Cyclophosphamide (CP)	Final dose: 80 mg/kg	Route: Oral Gavage

3. Test animals:

Species: Mice

Strain: Crl:CD-l®(JCR)BR

Age/weight at study initiation: 8 weeks at start of experiment

Source: Charles River Laboratories, I North Carolina.

No. animals used per dose (Rangefinder/Main Study)	**3** **6**	Males	**3** **0**	Females
Properly Maintained?	X	Yes		No

4. Test compound administration:

Study	Dose levels (mg/kg)	Final volume	Route
Dose range-finding	500, 1000, 1500, 2000	10 mL/kg	Oral gavage
Main study	0, 375, 750, 1500	10 mL/kg	Oral gavage

B. TEST PERFORMANCE:

1. Treatment and sampling times:

a. Test compound:

Dosing: 375, 750, 1500 mg/kg — [X] once [ ] twice (24 hrs apart) [ ] Other

Sampling (after last dose): [ ] 6 hr [ ] 12 hr [X] 24 hr [X] 48 hr [ ] 72 hr

b. Vehicle control:

Dosing: Corn oil — [X] once [ ] twice (24 hrs apart) [ ] Other

Sampling (after last dose): [ ] 6 hr [ ] 12 hr [X] 24 hr [X] 48 hr [ ] 72 hr

c. Positive control:

Dosing: 80 mg/kg — [X] once [ ] twice (24 hrs apart) [ ] Other

Sampling (after last dose) [ ] 6 hr [ ] 12 hr [X] 24 hr [ ] 48 hr [ ] 72 hr

2. Tissues and cells examined:

Bone marrow
No. of polychromatic erythrocytes (PCE) examined per animal: 2000
No. of normochromatic erythrocytes (NCE; more mature RBCs) examined per animal: 500
Other :

3. Details of slide preparation: Following centrifugation to pellet the marrow, the supernatant was removed by aspiration and portions of the pellet were spread on slides and air-dried. The slides were fixed in methanol, stained in May-Grunwald solution and Giemsa, and protected by mounting with coverslips. For control of bias, all slides were coded prior to analysis.

4. Evaluation criteria*:* The criteria for a positive response was the detection of a statistically significant increase in micronucleated PCEs for at least one dose level, and a statistically significant dose related response. A test article that did not induce both of these responses was considered negative.

The unit of scoring was the micronucleated cell, not the micronucleus; thus, the occasional cell with more than one micronucleus was counted as one micronucleated PCE, not two (or more) micronuclei.

Acceptance Criteria:

- The dose should reach the limit dose or produce some indication of toxicity, e.g., toxic signs and/or mortality in the test article dosed animals and/or a reduction in the PCE:NCE ratio.
- For the vehicle control group, the mean must be within the historical control range and be less than 0.4% micronucleated PCEs.
- The positive control group must show a statistically significant elevation of the mean of relative to the vehicle control group, and the positive control response must be consistent with historical positive control data.

5. Statistical methods: The following statistical methods were used to analyze the micronucleus data. Assay data analysis was performed using an analysis of variance on untransformed proportions of cells with micronuclei per animal and on untransformed PCE:NCE ratios when the variances were homogeneous. Ranked proportions were used for heterogeneous variances. If the analysis of variance was statistically significant ($p \leq 0.05$), Dunnett's t-test was used to determine which dose groups, if any, were statistically significantly different from the vehicle control. Analyses were performed separately for each sampling time. The low-, mid-, and high-dose groups, as well as the positive control group, were compared with the vehicle control group at the 5%, one-tailed probability level.

II. REPORTED RESULTS:

A. PRELIMINARY TOXICITY ASSAY: Mortality was observed in 2 males and 2 females at 2000 mg/kg. The remaining 2000 mg/kg animals were humanely sacrificed due to excessive toxicity in the dose group. Signs of clinical toxicity in the 2000 mg/kg animals included hypoactivity, squinted eyes, spasms, irregular respiration, recumbency, ataxia, hunched/flattened posture, crust-orbitals, and/or rough haircoat. Males exposed to 1500 mg/kg exhibited some of the same clinical signs of toxicity (squinted eyes, hypoactivity, flattened posture, irregular respiration, crust-orbitals- and rough coat) and a few animals from the 500 and 1000 mg/kg treatment groups (both sexes) exhibited minor clinical signs (slight hypoactivity and squinted eyes) 1 hour after dosing that resolved within a day. No mortalities were observed at doses ≤1500 mg/kg. Based on the results of the dose range finding assay, the maximum tolerated dose was estimated to be 1500 mg/kg.

Dose Range Finding Assay Table

Target Dose Level (mg/kg)	Stock Concentration (mg/mL)	Dosing Volume (mL/kg)	Route of Administration	Number of Animals	
				Male	Female
500	50	10	Oral gavage	3	3
1000	100	10	Oral gavage	3	3
1500	150	10	Oral gavage	3	-
2000	200	10	Oral gavage	3	3

From page 13 of study report

Table 1:
Mortality Summary – Dose Rangefinding Assay

Target Dose Level (mg/kg)	Number of Males (Died/Total Dosed)	Number of Females (Died/Total Dosed)
500	0/3	0/3
1000	0/3	0/3
1500	0/3	-
2000	3/3[a]	3/3[a]

[a] One animal in the dose group was humanely sacrificed due to excessive toxicity in the dose group.

From page 20 of study report

B. MICRONUCLEUS ASSAY:

Ethoxyquin did not induce statistically significant increases in micronucleated PCEs or changes in the PCE/NCE ratios or cytotoxic of the bone marrow at any test article dose examined (375, 750, and 1500 mg/kg). The vehicle control group and the group mean were within the historical control range. The positive control, cyclophosphamide, induced a statistically significant increase in micronucleated PCEs as compared to that of the vehicle control. Ethoxyquin, induced mortality in three 1500 mg/kg animals and showed signs of clinical toxicity (hypoactivity, squinted eyes, irregular respiration, ataxia, and/or a temporary trance) in the treated animals at 750 and 1500 mg/kg.

Table 3:
Mortality Summary – Micronucleus Assay

Target Dose Level (mg/kg)	Number of Males (Died/Total Dosed)
375	0/6
750	0/6
1500	3/18

From pg 22 of study report

Table 5: Micronucleus Assay – Summary Table

Assay No.: 25788-0-455OECD
Test Article: Ethoxyquin
Initiation of Dosing: 09 March 2004

Treatment	Dose	Harvest Time	% Micronucleated PCEs Mean of 2000 per Animal ± S.E. Males	Ratio PCE:NCE Mean ± S.E. Males
Controls				
Vehicle	Corn Oil 10mL/kg	24 hr	0.04 ± 0.01	0.69 ± 0.02
		48 hr	0.02 ± 0.01	0.69 ± 0.04
Positive	CP 80 mg/kg	24 hr	1.60 ± 0.08*	0.60 ± 0.04
Test Article	375 mg/kg	24 hr	0.05 ± 0.02	0.72 ± 0.10
	750 mg/kg	24 hr	0.03 ± 0.02	0.58 ± 0.07
	1500 mg/kg	24 hr	0.03 ± 0.02	0.56 ± 0.05
		48 hr	0.02 ± 0.01	0.54 ± 0.08

* Significantly greater than the corresponding vehicle control, $p \leq 0.01$.
CP = Cyclophosphamide
PCE = Polychromatic erythrocyte
NCE = Normochromatic erythrocyte
From page 24 of study report

III. DISCUSSION AND CONCLUSIONS:

A. **INVESTIGATORS' CONCLUSIONS:** The test article, ethoxyquin, was evaluated as negative in the mouse bone marrow micronucleus assay under the conditions of this assay.

B. REVIEWER COMMENTS:

The reviewer agrees with the investigator's findings. Clinical signs of toxicity including hypoactivity, squinted eyes, irregular respiration, ataxia and/or a temporary trance (standing on hind limbs with head in upright position with little movement) were observed in the 750 and 1500 mg/kg treatment groups and three mortalities was observed in the 1500 mg/kg group. Under the conditions of this assay, ethoxyquin did not elicit an increase in micronucleated PCEs in mouse bone marrow when tested up to 1500 mg/kg, a dose that induced overt toxicity, and is thus negative for micronuclei induction in mouse bone marrow.

C. STUDY DEFICIENCIES:

None noted

EPA Reviewer: Odbert Triplett **Signature**:
RAB VII Health Effects Division (7509P) **Date:** 09/18/19
EPA Secondary Reviewer: Austin Wray **Signature**:
RAB IV, Health Effects Division (7509P) **Date:** 09/18/19

Template version 09/11

TXR#: 0057942

DATA EVALUATION RECORD

STUDY TYPE: ***In vitro* Bacterial Gene Mutation***, Salmonella typhimurium; E. coli* mammalian activation gene mutation assay; OCSPP 870.5100[1] [§84-2]; OECD 471 (formerly OECD 471 & 472).

PC CODE: 055501 **DP BARCODE:** D453268

TEST MATERIAL (PURITY): Ethoxyquin (98.9% a.i.)

SYNONYMS: 6-Ethoxy- l,2-dihydro-2,2,4-tri methylquinoline,
1-2-Dihydro-6-ethoxy-2,2,4-trimethl quinoline

CITATION: Author Michael S. Mecchi, MS (26 July 2004). *Salmonella typhimurium; E. coli* Reverse Mutation gene mutation assay. Covance Laboratories Inc. 7529-102, 26 July 2004 MRID 46330701. Unpublished

SPONSOR: The Pear Bureau Northwest

EXECUTIVE SUMMARY: In a reverse gene mutation assay in bacteria (MRID 46330701), *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, and TA 1537, and *Escherichia coli* strain WP2uvrA were exposed to ethoxyquin (98.9% a.i., MRI Reference No.:8298:19-30) at concentrations of 6.67, 10, 33.3, 66.7, 100, 333, 667, 1000, 3330, and 5000 µg/plate in the presence and absence of mammalian metabolic activation using the plate incorporation procedure.

In the dose range finding assay cytotoxicity was observed with tester strain TA100 at 3330 µg/plate and above in the absence of S9 and at 667 µg/plate and above in the presence of S9 as evidenced by dose-related decreases in the number of revertants per plate and reduced bacterial background lawns. Cytotoxicity was also observed with tester strain WP211vrA at 5000 µg/plate in the presence and absence of S9 mix based on similar findings of dose-related decreases in the number of revertants per plate and reduced bacterial background lawns.

In the mutagenicity assay, cytotoxicity was observed in TA98, TA100, TA1535, and TA1537 at 5000 µg/plate in the absence of S9 and at 1000 µg/plate and above in the presence of S9. There were no marked increases in the mean number of revertants/plate in any strain in either trial of the mutagenicity assay. The positive controls induced marked increases in revertant colonies compared to controls in all strains in the presence and absence of S9 activation in both trials.

There was no evidence of induced mutant colonies over background due to exposure to

870.5100 - Reverse mutation *E. coli* WP2 and WP2uvrA; *S. typhimurium* TA 97, TA98, TA100, TA1535, TA1537

ethoxyquin up to cytotoxic concentrations.

This study is classified as **Acceptable/Guideline** and satisfies the guideline requirement (OCSPP 870.5100[1]; OECD 471) for *in vitro* mutagenicity (bacterial reverse gene mutation) data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS:

A. MATERIALS:

1. Test material:

Ethoxyquin

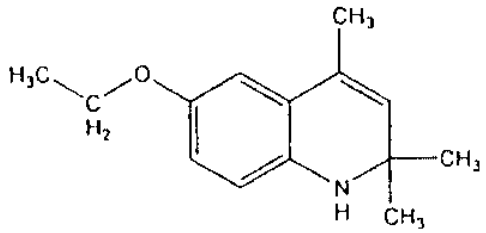
Description: Transparent, orange, viscous liquid

Lot/Batch #: 8298:19-30

Purity: 98.9%

CAS # of TGAI: 91-53-2

Structure:



Solvent Used: Dimethylsulfoxide (DMSO)

2. Control materials:

Vehicle: DMSO; study did not include a negative control.

Solvent (final conc): DMSO vehicle 50 uL/plate

Positive:

Nonactivation:

Sodium azide	2.0 µg/plate TA100, TA1535
2-Nitrofluorene	1.0 µg/plate TA98
Other:	
ICR-191	1.0 µg/plate TA1537
4-nitroauinoline-N-oxide	1.0 µg/plate WP2uvrA

Activation:

2-Aminoanthracene (2-anthramine)	2.5 µg/plate all strains except TA98
Other:	
benzo[a]pyrene	2.5 µg/plate TA98

3. Activation: S9 derived from

Liver microsomal enzymes (S9 homogenate) were purchased from Molecular Toxicology, Inc., Lot No 1615 (39.5 mg protein/mL) and Lot No. 1626 (41.5 mg protein/mL). The homogenate was prepared from male Sprague-Dawley rats that had been injected (i.p.) with Aroclor™ 1254 (200 mg/mL in corn oil) at 500 mg/kg as described by Ames *et al.,* (1975).

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Noninduced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other *(name)*
			Other *(name)*		Other *(name)*		

4. Test organisms: *S. typhimurium* and *E. coli* strains:

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538	X	WP2uvrA		
Properly maintained?						X	Yes		No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?						X	Yes		No

5. Test compound concentrations used:

Nonactivated conditions:
Salmonella typhimurium tester strains TA98, TA 100, TA1535, and TA1537: (10.0, 33.3, 100, 333, 1000, 2000, and 5000 ug/plate

Escherichia coli tester strain WP2uvrA : 33.3, 100, 333, 1000, 2000, and 5000 ug/plate

Activated conditions:
Salmonella typhimurium tester strains TA98, TA100, TA1535, and TA1537: (10.0, 33.3, 100, 333, 1000, 2000, and 5000 ug/plate

Escherichia coli tester strain WP2uvrA: 33.3, 100, 333, 1000, 2000, and 5000 ug/plate

In the mutagenicity assays, all concentrations of the test article, negative control, and positive controls were plated in triplicate, both in the presence and absence of S9 activation.

B. TEST PERFORMANCE:

1. Type of assay:

X	Standard plate test
	Pre-incubation ___ minutes
	"Prival" modification *(i.e. azo-reduction method)*
	Spot test
	Other *(describe)*

2. Protocol: Study was performed via duplicate trials. *Salmonella* and *E. coli* tester strains were exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and Maron and Ames (1983). When S9 mix was not required, 100 µL of tester strain and 50 µL of vehicle or test article dose were added to 2.5 mL of molten selective top agar. When S9 mix was required, 500 µL of S9 mix, 100 µL of tester strain and 50 µL of vehicle or test article dose were added to 2.0 mL of molten selective top agar. After the required components had been added, the mixture was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay solidified, the plates were inverted and incubated for 52 ± 4 hours at 37 ± 2°C. Positive control articles were plated using a 50 µL plating aliquot. Condition of the bacterial background lawn was evaluated for evidence of cytotoxicity and test article precipitate. Revertant colonies were counted by automated colony counter or by hand.

3. Statistical analysis: Mean revertants per plate and standard deviation was calculated for each replicate.

4. Evaluation criteria: For a test article to be considered positive, it had to produce at least a 2-fold increase in the mean revertants per plate of at least one of the tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate had to be accompanied by a dose response to increasing concentrations of the test article.

The assay was considered valid if the following criteria were met:

- All *S. typhimurium* tester strains exhibited sensitivity to crystal violet (*rfa* wall mutation),
- All tester strains exhibited sensitivity to ultraviolet light (*S. typhimurium* strains, *uvr*B mutation and *E. coli* strain, *uvr*A mutation),
- Strains TA98 and TA100 exhibited ampicillin resistance (pKM101 plasmid),
- The spontaneous reversion rates of all strains in the vehicle controls were within the historical control ranges,
- The positive controls induced at least a 3-fold increase in revertant colonies compared to the concurrent vehicle control, and
- A minimum of 3 non-toxic dose levels were available for evaluation.

b. Positive result: The test article was considered to be positive for mutagenicity if the following criteria were met:

- A dose-related, 2-fold or greater increase in the mean number of revertants/plate compared to solvent controls was observed in strains TA98, TA100, or WP2 *uvr*A.
- A dose-related, 3-fold or greater increase in the mean number of revertants/plate compared to solvent controls was observed in strains TA1535 or TA1537.

II. REPORTED RESULTS: The test material formulations were analyzed for actual concentrations using a gas chromatograph system (GC) with a flame ionization detector (FID). The measured concentrations for the dosing solution samples were 93.5-104% of nominal concentrations across the two trials of the mutagenicity assay. Formulations of 0.1 and 100 mg/mL in DMSO were shown to be stable for up to 7 days (96.4-102% nominal)

A. PRELIMINARY CYTOTOXICITY ASSAY:

In the dose range finding assay cytotoxicity was observed with tester strain TA100 at 3330 µg/plate and above in the absence of S9 and at 667 µg/plate and above in the presence of S9 as evidenced by dose-related decreases in the number of revertants per plate and reduced bacterial background lawns. Cytotoxicity was also observed with tester strain WP211vrA at 5000 µg/plate in the presence and absence of S9 mix based on similar findings of dose-related decreases in the number of revertants per plate and reduced bacterial background lawns.

B. MUTAGENICITY ASSAY:

The mutagenicity assay was performed in two duplicate trials, Trial 25788-B1 and 25788-C1. The mutagenicity results for the two trials are presented in Tables 2 through 7 on pages 21-26 of the study report. Copies of these tables are included in Appendix A of this DER.

Cytotoxicity was observed during the mutagenicity assay in TA98, TA100, TA1535, and

TA1537 at 5000 µg/plate in the absence of S9 and at 1000 µg/plate and above in the presence of S9. There were no marked increases in the mean number of revertants/plate in any strain in either trial. The positive controls induced marked increases in revertant colonies compared to controls in all strains in the presence and absence of S9 activation in both trials.

III. DISCUSSION AND CONCLUSIONS:

A. INVESTIGATORS' CONCLUSIONS:

The results of the *Salmonella-Escherichia* coli/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay indicate that under the conditions of this study, the test article, ethoxyquin, did not cause a positive increase in the mean number of revertants per plate with any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

B. REVIEWER COMMENTS:

Cytotoxicity (as indicated by thinning of background lawn and reduced number of revertants) was observed in TA100 at concentrations $\geq$ 3330 µg/plate in the absence of S9 mix and at concentrations $\geq$ 667 µg/plate in the presence of S9 during the dose range finding study, and in TA98, TA100, TA1535, and TA1537 at 5000 µg/plate in the absence of S9 and at 1000 µg/plate and above in the presence of S9 during the mutagenic assay .

There were no marked increases in the mean number of revertants/plate in any strain in the presence or absence of metabolic activation up to concentrations that were cytotoxic. The positive controls induced the appropriate response in both trials.

There was no evidence of induced mutant colonies over background due to exposure to ethoxyquin.

C. STUDY DEFICIENCIES:

None noted

Appendix A. Mutagenicity Assay Results Tables 2-7 from MRID 46330701

Table 2 : Mutagenicity Assay Results – Individual Plate Counts

Test Article ID: Ethoxyquin

Assay No.: 25788-0-409OECD Trial No.: B1

Date Plated: 09-Mar-04 Vehicle: DMSO

Date Counted: 16-Mar-04, 18-Mar-04 Plating Aliquot: 50 µL

		Revertants Per Plate												
	Dose/Plate	TA98			TA100			TA1535			TA1537			Background Lawn[a]
		1	2	3	1	2	3	1	2	3	1	2	3	
Microsomes: Rat Liver														
Vehicle Control		12	12	13	98	99	128	3	9	16	4	4	7	N
Test Article	10.0 µg	8	15	16	87	91	50	12	12	16	5	6	8	N
	33.3 µg	13	14	19	64	76	93	9	12	14	3	5	5	N
	100 µg	14	15	15	80	87	90	8	14	18	3	4	7	N
	333 µg	7	8	10	91	106	107	5	8	9	7	8	9	N
	1000 µg	9	9	14	69	103	112	7	8	8	4	5	6	N
	2000 µg	4	7	8	90	97	112	3	4	8	0	0	1	N
	5000 µg	0	2	1	11	14	14	1	1	2	0	0	1	R
Positive Control[b]		88	119	166	518	521	557	78	105	187	80	89	101	N
Microsomes: None														
Vehicle Control		13	16	21	77	79	84	14	15	15	5	8	10	N
Test Article	10.0 µg	9	10	16	79	80	87	14	14	17	1	5	5	N
	33.3 µg	10	13	15	67	72	93	16	17	24	5	9	10	N
	100 µg	15	23	24	76	77	69	10	13	21	2	3	5	N
	333 µg	14	21	31	80	97	111	9	12	14	4	5	10	N
	1000 µg	3	8	10	86	101	112	11	13	17	3	4	5	R
	2000 µg	0	4	5	10	15	16	1	4	5	0	0	2	R
	5000 µg	0	0	2	0	0	3	0	0	0	0	0	0	R
Positive Control[c]		181	202	260	913	1105	1231	624	726	732	733	758	774	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b]
TA98	benzo[a]pyrene	2.5 µg/plate
TA100	2-aminoanthracene	2.5 µg/plate
TA1535	2-aminoanthracene	2.5 µg/plate
TA1537	2-aminoanthracene	2.5 µg/plate

[c]
TA98	2-nitrofluorene	1.0 µg/plate
TA100	sodium azide	2.0 µg/plate
TA1535	sodium azide	2.0 µg/plate
TA1537	ICR-191	2.0 µg/plate

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Table 3 : Mutagenicity Assay Results – Summary

Test Article ID: Ethoxyquin

Assay No.: 25788-0-409OECD Trial No.: B1

Date Plated: 09-Mar-04 Vehicle: DMSO

Date Counted: 16-Mar-04, 18-Mar-04 Plating Aliquot: 50 µL

		Mean Revertants Per Plate with Standard Deviation								
	Dose/Plate	TA98		TA100		TA1535		TA1537		Background Lawn[a]
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Microsomes: Rat Liver										
Vehicle Control		12	1	108	17	9	7	5	2	N
Test Article	10.0 µg	13	4	76	23	13	2	6	2	N
	33.3 µg	15	3	78	15	12	3	4	1	N
	100 µg	15	1	86	5	13	5	5	2	N
	333 µg	8	2	101	9	7	2	8	1	N
	1000 µg	11	3	95	23	8	1	5	1	N
	2000 µg	6	2	100	11	5	3	0	1	N
	5000 µg	1	1	13	2	1	1	0	1	R
Positive Control[b]		124	39	532	22	123	57	90	11	N
Microsomes: None										
Vehicle Control		17	4	80	4	15	1	8	3	N
Test Article	10.0 µg	12	4	82	4	15	2	4	2	N
	33.3 µg	13	3	77	14	19	4	8	3	N
	100 µg	21	5	74	4	15	6	3	2	N
	333 µg	22	9	96	16	12	3	6	3	N
	1000 µg	7	4	100	13	14	3	4	1	R
	2000 µg	3	3	14	3	3	2	1	1	R
	5000 µg	1	1	1	2	0	0	0	0	R
Positive Control[c]		214	41	1083	160	694	61	755	21	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b]
TA98	benzo[a]pyrene	2.5 µg/plate
TA100	2-aminoanthracene	2.5 µg/plate
TA1535	2-aminoanthracene	2.5 µg/plate
TA1537	2-aminoanthracene	2.5 µg/plate

[c]
TA98	2-nitrofluorene	1.0 µg/plate
TA100	sodium azide	2.0 µg/plate
TA1535	sodium azide	2.0 µg/plate
TA1537	ICR-191	2.0 µg/plate

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Table 4 : Mutagenicity Assay Results – Individual Plate Counts and Summary

Test Article ID: Ethoxyquin

Assay No.: 25788-0-409OECD Trial No.: B1

Date Plated: 09-Mar-04 Vehicle: DMSO

Date Counted: 16-Mar-04, 18-Mar-04 Plating Aliquot: 50 µL

	Dose/Plate	Revertants Per Plate WP2*uvr*A			Mean Revertants Per Plate with Standard Deviation WP2*uvr*A		Background Lawn[a]
		1	2	3	Mean	S.D.	
Microsomes: Rat Liver							
Vehicle Control		24	26	29	26	3	N
Test Article	33.3 µg	21	23	25	23	2	N
	100 µg	17	21	23	20	3	N
	333 µg	14	16	22	17	4	N
	1000 µg	10	11	19	13	5	N
	3330 µg	11	11	12	11	1	N
	5000 µg	3	4	8	5	3	R
Positive Control[b]		497	527	585	536	45	N
Microsomes: None							
Vehicle Control		12	15	19	15	4	N
Test Article	33.3 µg	16	17	29	21	7	N
	100 µg	10	12	19	14	5	N
	333 µg	9	17	21	16	6	N
	1000 µg	21	21	24	22	2	N
	3330 µg	7	9	10	9	2	N
	5000 µg	3	5	6	5	2	R
Positive Control[c]		161	182	197	180	18	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b] WP2*uvr*A 2-aminoanthracene 25.0 µg/plate [c] WP2*uvr*A 4-nitroquinoline-N-oxide 1.0 µg/plate

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Table 5 : Mutagenicity Assay Results – Individual Plate Counts

Test Article ID: Ethoxyquin

Assay No.: 25788-0-409OECD Trial No.: C1

Date Plated: 07-Apr-04 Vehicle: DMSO

Date Counted: 12-Apr-04 Plating Aliquot: 50 µL

	Dose/Plate	Revertants Per Plate												Background Lawn[a]
		TA98			TA100			TA1535			TA1537			
		1	2	3	1	2	3	1	2	3	1	2	3	
Microsomes: Rat Liver														
Vehicle Control		23	24	22	71	91	94	9	6	5	9	6	9	N
Test Article	10.0 µg	19	24	23	82	97	88	6	11	10	7	2	7	N
	33.3 µg	17	23	27	103	82	93	7	C	8	4	12	4	N
	100 µg	24	19	22	93	90	95	6	C	9	7	9	5	N
	333 µg	21	18	22	102	99	95	5	7	8	8	14	5	N
	1000 µg	10	20	19	93	89	67	12	12	10	1	0	C	N/R[d]
	2000 µg	4	15	14	19	19	23	2	2	7	0	0	0	R
	5000 µg	0	2	3	1	0	0	1	1	0	0	0	0	R
Positive Control[b]		174	173	168	430	534	373	95	88	82	93	112	104	N
Microsomes: None														
Vehicle Control		13	9	9	67	78	71	11	10	9	4	12	6	N
Test Article	10.0 µg	8	13	13	69	71	63	11	14	5	2	5	6	N
	33.3 µg	13	5	9	77	85	71	10	5	8	3	C	3	N
	100 µg	8	11	8	78	68	102	11	12	7	1	3	2	N
	333 µg	8	13	10	69	82	80	11	6	11	6	1	2	N
	1000 µg	4	7	2	76	76	88	8	10	C	2	7	2	R
	2000 µg	1	3	0	6	9	3	1	0	3	0	1	2	R
	5000 µg	0	0	0	8	0	0	1	0	0	0	0	0	R
Positive Control[c]		174	169	147	1145	1136	1072	636	707	601	1169	1422	1147	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b] TA98 benzo[a]pyrene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate
TA1535 2-aminoanthracene 2.5 µg/plate
TA1537 2-aminoanthracene 2.5 µg/plate

[c] TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate
TA1535 sodium azide 2.0 µg/plate
TA1537 ICR-191 2.0 µg/plate

C = No count due to contamination on the plate.
[d] The first entry is the lawn evaluation for tester strains TA98, TA1535, and TA1537.
The second entry is the lawn evaluation for tester strain TA100.

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Table 6 : Mutagenicity Assay Results – Summary

Test Article ID: Ethoxyquin

Assay No.: 25788-0-409OECD Trial No.: C1

Date Plated: 07-Apr-04 Vehicle: DMSO

Date Counted: 12-Apr-04 Plating Aliquot: 50 µL

		Mean Revertants Per Plate with Standard Deviation								
	Dose/Plate	TA98		TA100		TA1535		TA1537		Background Lawn[a]
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Microsomes: Rat Liver										
Vehicle Control		23	1	85	13	7	2	8	2	N
Test Article	10.0 µg	22	3	89	8	9	3	5	3	N
	33.3 µg	22	5	93	11	8	1	7	5	N
	100 µg	22	3	93	3	8	2	7	2	N
	333 µg	20	2	99	4	7	2	9	5	N
	1000 µg	16	6	83	14	11	1	1	1	N/R[d]
	2000 µg	11	6	20	2	4	3	0	0	R
	5000 µg	2	2	0	1	1	1	0	0	R
Positive Control[b]		172	3	446	82	88	7	103	10	N
Microsomes: None										
Vehicle Control		10	2	72	6	10	1	7	4	N
Test Article	10.0 µg	11	3	68	4	10	5	4	2	N
	33.3 µg	9	4	78	7	8	3	3	0	N
	100 µg	9	2	83	17	10	3	2	1	N
	333 µg	10	3	77	7	9	3	3	3	N
	1000 µg	4	3	80	7	9	1	4	3	R
	2000 µg	1	2	6	3	1	2	1	1	R
	5000 µg	0	0	3	5	0	1	0	0	R
Positive Control[c]		163	14	1118	40	648	54	1246	153	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b] TA98	benzo[a]pyrene	2.5 µg/plate	[c] TA98	2-nitrofluorene	1.0 µg/plate
TA100	2-aminoanthracene	2.5 µg/plate	TA100	sodium azide	2.0 µg/plate
TA1535	2-aminoanthracene	2.5 µg/plate	TA1535	sodium azide	2.0 µg/plate
TA1537	2-aminoanthracene	2.5 µg/plate	TA1537	ICR-191	2.0 µg/plate

[d] The first entry is the lawn evaluation for tester strains TA98, TA1535, and TA1537. The second entry is the lawn evaluation for tester strain TA100.

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Table 7 : Mutagenicity Assay Results – Individual Plate Counts and Summary

Test Article ID: Ethoxyquin

Assay No.: 25788-0-409OECD Trial No.: C1

Date Plated: 07-Apr-04 Vehicle: DMSO

Date Counted: 12-Apr-04 Plating Aliquot: 50 µL

	Dose/Plate	Revertants Per Plate WP2*uvr*A			Mean Revertants Per Plate with Standard Deviation WP2*uvr*A		Background Lawn[a]
		1	2	3	Mean	S.D.	
Microsomes: Rat Liver							
Vehicle Control		12	13	14	13	1	N
Test Article	33.3 µg	14	12	15	14	2	N
	100 µg	13	4	10	9	5	N
	333 µg	16	15	18	16	2	N
	1000 µg	12	28	11	17	10	N
	3330 µg	7	9	11	9	2	N
	5000 µg	9	9	3	7	3	N
Positive Control[b]		246	220	291	252	36	N
Microsomes: None							
Vehicle Control		10	8	14	11	3	N
Test Article	33.3 µg	11	13	14	13	2	N
	100 µg	10	9	13	11	2	N
	333 µg	13	11	15	13	2	N
	1000 µg	6	19	10	12	7	N
	3330 µg	11	9	9	10	1	N
	5000 µg	7	10	14	10	4	R
Positive Control[c]		235	198	197	210	22	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b] WP2*uvr*A 2-aminoanthracene 25.0 µg/plate [c] WP2*uvr*A 4-nitroquinoline-N-oxide 1.0 µg/plate
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EPA Reviewer: Odbert Triplett **Signature:**
RABVII Health Effects Division (7509P) **Date:** 09/18/19
EPA Secondary Reviewer: Austin Wray **Signature**:
RAB IV, Health Effects Division (7509P) **Date:** 09/18/19

Template version 09/11

TXR#: 0057942

DATA EVALUATION RECORD

STUDY TYPE: ***In vitro* Mammalian Cytogenetics:** *In Vitro* Mammalian Chromosome Aberration Test OCSPP 870.5375 [§84-2]; OECD 473

PC CODE: 055501 **DP BARCODE:** D453268

TEST MATERIAL (PURITY): Ethoxyquin (98.93% a.i.)

SYNONYMS: 6-Ethoxy- l,2-dihydro-2,2,4-tri methylquinoline,
1-2-Dihydro-6-ethoxy-2,2,4-trimethl quinoline

CITATION: H. Murli. 2004. Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells. Covance Laboratories Inc. Vienna, VA. Study # 7529-109. August 6, 2004. MRID 46338901. Unpublished

SPONSOR: Pear Bureau Northwest, 4382 S.E. International Way, Suite A Milwaukie, Oregon 97222-4635

EXECUTIVE SUMMARY:

In a mammalian cell chromosomal aberration study (MRID 46338901), CHO cell cultures were exposed to ethoxyquin (98.93% a.i., batch/lot #A018490001) in DMSO in two separate assays.

In the initial assay, CHO cells were exposed for 3 hours at concentrations of 0, 6.78, 9.69, 13.8, 19.8, 28.2, 40.4, 57.6, 82.4, 118, 168, 240, 343, 490, 700, and 1000 µg/mL with and without metabolic activation and harvested 20 hours after treatment. The results from the metabolic activated cultures treated with 13.8, 19.8, 28.2, 40.4, 57.6, 82.4, 118, and 168 µg/mL show mitotic indices were reduced by 53%, 47%, 69%, 86%, 72%, 70%, 77%, and 98% respectively. No cell monolayer was observed on slides prepared from cultures treated with ≥240 µg/mL. Chromosomal aberrations were assessed in the metabolically activated cultures treated with 9.69, 13.8, 19.8 and 28.2 µg/mL and significant increases in cells with chromosomal aberrations was observed at all concentrations assessed. In addition, polyploidy was evident in the 13.8 µg/mL treatment group and a significant increase in endoreduplication was observed in the cells treated with 9.69 and 13.8 µg/mL.

The mitotic indices for the cultures without metabolic activation exhibited a reduction of 28%, 35%, 53%, and 86% at concentrations of 40.4, 57.6, 82.4, and 118 µg/mL, respectively. No cell monolayer was observed on slides prepared from cultures treated with ≥168 µg/mL. Chromosomal aberrations in the absence of metabolic activation were analyzed in cultures

treated with 28.2, 40.4, 57.6, and 82.4 µg/mL. A significant increase in chromosomal aberrations and polyploidy were observed in cells treated with 82.4 µg/ml. Significant increases in endoreduplication were observed in the cultures treated with 28.2, 57.6, and 82.4 µg/mL.

In the confirmatory assay, CHO cells were exposed for 3 hours at concentrations of 5.00, 10.0, 15.0, 20.0, 25.0, 30.0, 40.0, and 50.0 µg/mL with metabolic activation or for 20 hours at concentrations of 1.25, 2.50, 5.00, 10.0, 15.0, 20.0, 30.0, 50.0, 75.0, and 100 µg/mL without metabolic activation and then harvested 20 hours after treatment. Cultures with metabolic activation exposed to 10.0, 15.0, 20.0, 25.0, 30.0, 40.0, and 50.0 µg/mL showed a change in mitotic indices of 15%, 56%, 53%, 65%, 79%, 78%, and 74% compared to controls. Although dead cells were noted on slides prepared from cultures treated with ≥15 µg/mL, the cell monolayer was described as healthy. Chromosomal aberrations in the presence of metabolic activation were assessed in cultures treated with 10.0, 15.0, 20.0, and 25.0 µg/mL. A significant increase in cells with chromosomal aberrations was observed in cultures treated with 15.0, 20.0, and 25.0 µg/ml. In addition, a significant increase in polyploidy was observed in the cultures treated with 20.0 µg/mL ethoxyquin and a significant increase in endoreduplication was observed in the cultures treated with 10.0, 15.0, and 20.0 µg/mL ethoxyquin.

Reductions of 0%, 0%, 37%, 46%, 54%, 96%, 100%, and 100% were observed in the mitotic indices of the cultures treated with 5.00, 10.0, 15.0, 20.0, 30.0, 50.0, 75.0, and 100 µg/mL, respectively, as compared with the vehicle control cultures in the assay without metabolic activation. No cell monolayer was observed on slides prepared from cultures treated with ≥75 µg/mL. Chromosomal aberrations in the absence of metabolic activation were assessed in cultures treated with 10.0, 15.0, 20.0, and 30.0 µg/mL of ethoxyquin. A significant increase in cells with chromosomal aberrations was observed in the cultures treated with 20.0 and 30.0 µg/mL. A significant increase in polyploidy was observed in the cultures treated with 30.0 µg/mL. No significant increase in endoreduplication was observed in the cultures analyzed.

There was evidence of chromosome aberration induced over background. The test article, ethoxyquin, is considered positive for inducing structural chromosomal aberrations in CHO cells in the presence and absence of metabolic activation.

This study is classified as **Acceptable/Guideline** and satisfies the guideline requirement (OCSPP 870.5375; OECD 473) for *in vitro* cytogenetic mutagenicity data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

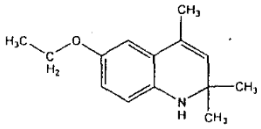
I. MATERIALS AND METHODS:

A. MATERIALS:

1. Test material: Ethoxyquin

Description: Orange, viscous, transparent liquid
Lot/Batch/MRI Reference #: 8298: 19-30
Purity: 98.93% a.i.
CAS # of TGAI: 91-53-2

Solvent used: Dimethylsulfoxide DMSO
Structure:



2. Control materials:

Negative control: McCoy's 5a culture medium

Solvent control (final conc'n): Dimethylsulfoxide (DMSO) 10.0 µL/mL

Positive control: Nonactivation: Mitomycin C 0.750 and 1.50 µg/ml in water (initial assay)
0.2 and 0.4 µg/ml in water (confirmatory)

Activation: Cyclophosphamide 7.50 and 12.5 µg/ml in water

3. Activation: S9 derived from

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Non induced		Phenobarbital		Mouse		Lung
			None		Hamster		Other *(name)*
			Other *(name)*		Other *(name)*		

Describe S9 mix composition: The *in vitro* metabolic activation system (Maron and Ames, 1983) consisted of a rat liver post-mitochondrial fraction (S9) and an energy-producing system of NADP plus isocitric acid. (Molecular Toxicology, Inc., Lot No. 1569)

4. Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
	Human lymphocytes
X	Chinese hamster ovary (CHO) cells

Media:

Properly maintained?	X	Yes		No
Periodically checked for *Mycoplasma* contamination?	X	Yes		No
Periodically checked for karyotype stability?	X	Yes		No

5. Test compound concentrations used:

Initial assay:
Without metabolic activation concentrations: 6.78, 9.69, 13.8, 19.8, 28.2, 40.4, 57.6, 82.4, 118, 168, 240, 343, 490, 700, and 1000 µg/mL

Metabolic activation concentrations: 6.78, 9.69, 13.8, 19.8, 28.2, 40.4, 57.6, 82.4, 118, 168, 240, 343, 490, 700, and 1000 µg/mL

Confirmatory assay:
Without metabolic activation concentrations: 1.25, 2.50, 5.00, 10.0, 15.0, 20.0, 30.0, 50.0, 75.0, and 100 ug/mL

Metabolic activation concentrations: 5.00, 10.0, 15.0, 20.0, 25.0, 30.0, 40.0, and 50.0 µg/mL

B. TEST PERFORMANCE:

1. Cytogenetic assay:

a. Cell exposure time:

	Test material	Solvent control	Positive control
Non-activated: Initial/Confirmatory	3/20 hours	3/20 hours	3/20 hours
Activated: Initial/Confirmatory	3/3 hours	3/3 hours	3/3 hours

b. Spindle inhibition:

Inhibition used/concentration:	0.1 ug/ml of Colcemid®
Administration time:	2 hours before cell harvest (~18 hours)

c. Cell harvest time after termination of treatment:

	Test material	Solvent control	Positive control
Non-activated:	20h	20h	20h
Activated:	20h	20h	20h

d. Details of slide preparation: Slides were prepared by dropping the harvested cultures on clean, glass slides and air-dried. The slides were stained with 5% Giemsa solution for the analysis of mitotic index and chromosomal aberrations. All slides were then air-dried and permanently mounted.

e. Metaphase analysis

No. of cells examined per dose: 100 cells/dose

Scored for structural?	X	Yes		No
Scored for numerical?	X	Yes , (*polyploid, endoreduplicated cells)*		No
Coded prior to analysis?	X	Yes		No

f. Evaluation criteria:

Acceptable Controls. The negative (untreated) and vehicle control cultures must contain less than approximately 5% cells with aberrations. The positive control result must be significantly higher (p >0.0 l) than the vehicle controls.

Acceptable High Dose. If the aberration results are negative and there is no significant reduction (approximately ≥50%) in mitotic index, the assay must include the highest applicable dose (a target dose of 10 mM or 5 mg/mL, whichever is lower) or a dose exceeding the solubility limit in culture medium.

Acceptable Number of Doses. The assay must include at least three analyzable concentrations.

After completion of microscopic analysis, data were decoded. The following factors are considered in evaluation of the test article data:

- The number and percentages of aberrant cells excluding gaps (-g).
- The number and percentages of aberrant cells including gaps (+g).
- Evidence of a dose-response relationship.

After completion of microscopic analysis, data will be decoded. The following factors were considered in evaluation of the test article data:

- A test article will be considered positive for inducing chromosomal aberrations if a significant increase ($p \leq 0.01$) in the number of cells with chromosomal aberrations is observed at one or more dose levels.

g. Statistical analysis: Statistical analysis employed a Cochran- Armitage test for linear trend and Fisher's Exact Test (Thakur et al., 1985) to compare the percentage of cells with aberrations in treated cells to the results obtained for the vehicle controls.

Statistical analysis was also performed for cells exhibiting polyploidy and/or endoreduplication in order to indicate significant ($p \leq 0.01$) increases in these events as indicators of possible induction of numerical aberrations; however, the test articles were evaluated only for structural aberrations and not for numerical aberrations by this protocol.

II. REPORTED RESULTS:

Ethoxyquin was immiscible/insoluble in cell culture grade water at ~88. 7 mg/mL. using concentrations of 1910 and 3810 ug/mL, the test article precipitated as droplets. At 955 µg/mL, a precipitate of fine-sized droplets was observed. The highest concentration tested in the assay, 1000 µg/mL, was above the solubility limit of the test article after dosing into culture medium.

Initial Assay:
The treatment period was 3 hours with and without metabolic activation and cultures were harvested ~20 hours from the initiation of treatment.

In the assay without metabolic activation reductions of 28%, 35%, 53%, and 86% were observed in the mitotic indices of the cultures treated with 40.4, 57.6, 82.4, and 118 µg/mL. Due to severe toxicity, only dead cells were observed on the slides prepared from the cultures treated with ≥168 µg/mL. Chromosomal aberrations were analyzed from the cultures treated

with 28.2, 40.4, 57.6, and 82.4 µg/mL. A significant increase in cells with chromosomal aberrations and polyploidy was observed in the cultures treated with 82.4 µg/mL. A significant increase in endoreduplication was observed in the cultures treated with 28.2, 57.6, and 82.4 µg/mL. A precipitate was observed prior to washing cultures at concentrations ≥ 343 µg/mL, and at concentrations ≥ 240 µg/mL prior to harvest.

In the assay with metabolic activation, reductions of 53%, 47%, 69%, 86%, 72%, 70%, 77%, and 98% were observed in the mitotic indices of the cultures treated with 13.8, 19.8, 28.2, 40.4, 57.6, 82.4, 118, and 168 µg/mL, respectively. Severe toxicity was observed on the slides prepared from the cultures treated with ≥240 µg/mL. Chromosomal aberrations were analyzed from the cultures treated with 9.69, 13.8, 19.8, and 28.2 µg/mL. A significant increase in cells with chromosomal aberrations was observed in all of the concentration levels analyzed. A significant increase in polyploidy was observed in the cultures treated with 13.8 µg/mL. A significant increase in endoreduplication was observed in the cultures treated with 9.69 and 13.8 µg/mL. A precipitate was observed after dosing at ≥ 240 µg/mL, prior to wash of the cultures treated with ≥ 57.6 µg/mL, and prior to harvest of the cultures treated with ≥ 343 µg/mL.

Confirmatory assay:
In a confirmatory chromosomal aberrations assay, the treatment period was for ~20 hours without metabolic activation and 3 hours with metabolic activation and the cultures were harvested ~20 hours from the initiation of treatment. There was no observed precipitate at any concentration with and without metabolic activation during these assays.

In the assay without metabolic activation, reductions of 0%, 0%, 37%, 46%, 54%, 96%, 100%, and 100% were observed in the mitotic indices of the cultures treated with 5.00, 10.0, 15.0, 20.0, 30.0, 50.0, 75.0, and 100 µg/mL, respectively, as compared with the vehicle control cultures in the assay without metabolic activation. No cell monolayer was observed on slides prepared from cultures treated with ≥75 µg/mL. Chromosomal aberrations were analyzed from the cultures treated with 10.0, 15.0, 20.0, and 30.0 µg/mL ethoxyquin. A significant increase in cells with chromosomal aberrations was observed in the cultures treated with 20.0 and 30.0 µg/mL. A significant increase in polyploidy was observed in the culture treated with 30.0 µg/mL. No significant increase in endoreduplication was observed in the cultures without metabolic activation.

In the assay with metabolic activation, cultures exposed to 10.0, 15.0, 20.0, 25.0, 30.0, 40.0, and 50.0 µg/mL showed a change in mitotic indices of 15%, 56%, 53%, 65%, 79%, 78%, and 74% compared to controls. Although dead cells were noted on slides prepared from cultures treated with ≥15 µg/mL, the cell monolayer was described as healthy. Chromosomal aberrations were analyzed from the cultures treated with 10.0, 15.0, 20.0, and 25.0 µg/mL ethoxyquin. Significant increases in cells with chromosomal aberrations was observed in the cultures treated with 15.0, 20.0, and 25.0 µg/ml ethoxyquin. A significant increase in polyploidy was observed in the cultures treated with 20.0 µg/mL ethoxyquin. Significant increase in endoreduplication was observed in the cultures treated with 10.0, 15.0, and 20.0 µg/mL ethoxyquin.

For the confirmatory assays the concentrations for the metabolic activated mentioned above were consistent with historical control data for negative and positive controls.

The sensitivity of the cell cultures for induction of chromosomal aberrations was shown by the increased frequency of aberrations in the cells exposed to mitomycin C, the positive control agent under nonactivated conditions and the successful activation by the metabolic system was illustrated by the increased incidence of cells with chromosomal aberrations in the cultures induced with cyclophosphamide, the positive control agent.

III. DISCUSSION AND CONCLUSIONS:

A. INVESTIGATORS' CONCLUSIONS:

The test article, ethoxyquin, was considered positive for inducing structural chromosomal aberrations in CHO cells with and without metabolic activation

B. REVIEWER COMMENTS:

The reviewer agrees with the investigator's conclusions. Ethoxyquin elicited an increase in chromosomal aberrations, polyploidy, and endoreduplication in CHO cells in the presence and absence of metabolic activation. These findings were observed in two separate assays and at concentrations below the solubility limit.

C. STUDY DEFICIENCIES:

None